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Polymerase mutations rtN238R, rtT240Y and rtN248H of hepatitis B virus decrease susceptibility to adefovir

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Long term antiviral therapy with nucleos(t)ide analogs (NAs) may lead to the emergence of drug-resistance viral mutants in chronic hepatitis B virus (HBV) patient. The purpose of this study was to identify adefovir dipivoxil (ADV) resistance mutations of HBV polymerase and determine effective drugs to replace ADV. The reverse transcriptase (RT) coding region was PCR-amplified using HBV DNA extracted from patient blood samples and sequenced. Nineteen substitution mutations were detected. Among them, rtN238R, rtT240Y and rtN248H were often observed in patients receiving ADV administration. These three potential drug resistant sites were introduced into HBV replication-competent plasmids. The *in vitro* susceptibility of both wild-type (WT) and mutant-type (MT) HBV to NAs was analyzed by Southern blotting and quantitative real-time PCR. The rtN238R, rtT240Y and rtN248H substitutions had no obvious effect on HBV DNA replication or gene expression. The *in vitro* susceptibility analysis showed that rtN238R, rtT240Y and rtN248H substitutions were responsible for the reduced susceptibility to ADV, and demonstrated a 5.42-, 2.89- and 5.72-fold increase in resistance towards ADV, respectively. However, HBV harbored these mutations retained normal susceptibility to LMV, LdT, ETV and TDF.

hepatitis B virus, resistance mutation, adefovir dipivoxil (ADV)

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Hepatitis B virus (HBV) is a member of the hepadnavirus family and contains a 3.2 kb, relaxed circular (RC), partially double-stranded DNA genome [1,2] with four overlapping open reading frames (ORFs): P, S, C, and X [3]. HBV infection can lead to acute and chronic necroinflammatory liver diseases, especially hepatocellular carcinoma (HCC), which account for half a million to 1.2 million deaths each year in the more than 350 million people who are chronic carriers of HBV [4–7]. HBV replicates through reverse transcription of an RNA intermediate, called pregenomic RNA (pgRNA) [8]. Like retroviruses with an error-prone polymerase, such as human immunodeficiency virus (HIV), HBV has a quasispecies distribution in infected individuals [4,9]; virions with relevant resistance mutations can be selected after treatment with nucleos(t)ide analogue (NA) drugs.

To date, complete HBV eradication is seldom accomplished with currently available agents such as interferon and NAs [10]. Lamivudine (LMV), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT) and tenofovir (TDF) are widely used for treatment of chronic hepatitis B (CHB) [11]. NA treatment inhibits HBV replication in patients and significantly reduces mortality, the risk of cirrhosis-related complications, and the incidence of hepatocellular carcinoma (HCC) [12]. However, the frequent emergence of drug-resistance mutations often leads to treatment failure and liver disease progression [13].

Various drug-resistant mutations of HBV have been identified, all of which were located in the reverse tran-

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scriptase (RT) region of HBV polymerase, which is further divided into seven domains: A–G [14]. Resistance to LMV and LdT is conferred by mutations in the YMDD motif within the C domain. The most common mutations are rtM204I/V [15], which are usually accompanied with compensatory mutations of rtL180M and/or rtV173L to restore HBV replication capacity [12,16,17]. An rtA181V/T or rtN236T substitution can reduce the anti-HBV effect of ADV [18]. Resistance to ETV requires a combination of mutations in the B, C, or D domain, such as rtI169T, rtM250I/V, and background substitutions at position rt204 [12,19]. However, there has been no reported TDF resistance mutation, except rtA194T which needs further confirmation [12,20–22].

The present study focused on the analysis of HBV polymerase sequence variation from CHB patients to detect novel potential drug-resistance mutations. Analysis of HBV sequences from 5 CHB patients undergoing LMV and ADV alternate therapies identified many substitution mutations, including rtN238R, rtT240Y and rtN248H. These newly discovered mutations were then introduced into HBV replication-competent plasmids. *In vitro* analysis demonstrated that rtN238R, rtT240Y and rtN248H resulted in 5.61-, 2.76- and 5.71-fold increases in resistance toward ADV, respectively. The results of the present study should provide certain specific references for clinical drug administration.

1 Mateirals and methods

(i) CHB patients and sequence analysis. Serum samples from CHB patients receiving NA therapy were collected, from which HBV DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The HBV-RT segment was PCR-amplified with the primer pair: RT-F and RT-R (Table 1), and cloned into the pCR2.1 vector. Five clones of each PCR product were two-way sequenced and aligned with WT HBV (GenBank accession no. X02763.1, genome type A, subtype *adw*2).

Table 1 Primer sequences for the construction of MT HBV plasmids and qRT-PCR[a)]

Name	Sequence (5′→3′)	Amplicon size (bp)
RT-F	CTAGGACCCCTGCTCGTGTT	843
RT-R	CGCAAACCCCAAAAGACCCA	
Fusion-F	TCTTCTCGAGGATTGGGGACC	1257
Fusion-R	GCAGCCATGGAAACGATGTAT	
rtN238R-F	CATTTA AACCCT***CG***CAAAACA AAG	552
rtN238R-R	CTTTGTTTTG***CG***AGGGTTTAAATG	730
rtT240Y-F	TAAACCCTAACAAA***TAC***AAGAGATG	548
rtT240Y-R	CATCTCTT***GTA***TTTGTTAGGGTTTA	735
rtN248H-F	GGGTTACTCTCTA***C***ATTTTATGGGT	523
rtN248H-R	CATAAAAT***G***TAGAGAGTAACCCCTA	760
HBV-RC-F	GTTGCCCGTTTGTCCTCTAATTC	100
HBV-RC-R	GGAGGGATACATAGAGGTTCCTT	

a) MT, mutant; qRT-PCR, quantitative real-time PCR; F, forward; R, reverse; bold text, italics: substitution mutation for mutant HBV plasmids construction.

(ii) Plasmid constructs. HBV mutants (MTs) were constructed using fusion-PCR with the primers listed in Table 1 carrying aimed mutations and pHBV1.3 as a template, which is a replication-competent plasmid including 1.3-fold over-length HBV genotype A2 genome (GenBank accession no. X02763.1, subtype *adw*2) [23,24], with a pBluescript KS+backbone.

(iii) NAs. NAs including 2′,3′-dideoxy-3′-thiacytidine (LMV; Glaxo Smith Kline, Brentford, Middlesex, UK), ADV (Gilead Sciences, Foster City, CA, USA), ETV (Bristol-Myers Squibb Co., New York, NY, USA), LdT (Novartis Pharmaceuticals Canada Inc., Dorval, Quebec, Canada) and TDF (Gilead Sciences) were diluted according to the manufactures' instructions and used in the assays at the indicated concentrations.

(iv) Cell culture and transfection. Hepatocellular carcinoma Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 atmosphere supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 2 mmol L^{-1} of glutamine, 100 IU mL^{-1} of penicillin and 100 IU m L^{-1} of streptomycin [25]. Huh7 cells were seeded in 6-well plates (1×10^6/well) and transfected with 2 μg HBV-bearing plasmid per well using lipofectamine 2000 (Invitrogen) with or without the indicated NA concentrations. For transfection experiments, HBV-bearing plasmids were co-transfected with a SEAP expression vector into Huh7 cells, the relative efficiency of transfections was assessed by measuring SEAP activity in the culture medium using a chemiluminescent detection method [26].

(v) Enzyme-linked immunosorbent assay (ELISA). Huh7 cells were transfected with the indicated HBV-bearing plasmids, hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in the supernatant at 96 h post transfection (hpt) were detected using a diagnostic kit (Shanghai Kehua Diagnostic Medical Products Co., Ltd., Shanghai, China) according to the manufacturer's instructions [25].

(vi) Western blotting. Huh7 cells were transfected with the indicated HBV-bearing plasmids and harvested at 96 hpt. The protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Total cell lysates (50 μg) from the transfected cells were assayed by Western blotting by probing with an anti-hepatitis B core antigen (HBcAg) antibody (Dako Cytomation Co., Carpinteria, CA, USA) and anti-*β-actin* (Beyotime Biotech Inc., Nantong, China), and detected by chemiluminescence (SuperSignal West Pico; Thermo Scientific-Pierce Protein Biology Products, Rockford, IL, USA) followed by autoradiography and densitometry analysis using a secondary goat anti-mouse IgG HRP antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; diluted 1∶10000) [27,28].

(vii) Analysis of HBV DNA from intracellular core particles by Southern blotting. Replication competent HBV WT/MTs plasmids were transfected into Huh7 cells. Total HBV DNA replicative intermediates from intracellular core particles were extracted and subjected to agarose gel electrophoresis, followed by denaturation and Southern blotting with a ^{32}P-labeled full length HBV probe according to previously published protocols [25,29]. Hybridization signals were visualized and analyzed using a phosphoimager (Cyclone, Packard Instrument Co., Inc., Meriden, CT, USA). Data were quantified with OptiQuant software (Packard Bioscience Co., Inc., Meriden, CT, USA).

(viii) Quantitative real-time PCR (qRT-PCR). Cell lysates of Huh7 were treated with DNase I (Roche) at 37°C, 30 min to digest input plasmid DNA. Total HBV DNA replicative intermediates were purified from lysates of Huh7 cells 96 hpt and were used as templates for qRT-PCR, which was conducted using SYBR Green I nucleic acid stain (Roche Diagnostics GmbH, Mannheim, Germany) on a Light Cycler real-time thermal cycler (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions [29]. Primers HBV-RC-F and HBV-RC-R (Table 1) hybridized to the HBV surface gene were designed to quantify HBV-DNA RC genomes (100 bp fragment) by qRT-PCR relative to an external plasmid DNA standard [30].

(ix) Statistical analysis. The statistical analysis was carried out using GraphPad (GraphPad Software, San Diego, USA). Differences in multiple comparisons were determined for statistical significance using Student's *t*-test. $P<0.05$ was considered as statistically significant. Results are presented as means ± SD.

2 Results

2.1 Sequence analysis of HBV-RT from CHB patients

Five patients with lengthy CHB histories (>3 years) took part in this study. An increase in serum alanine aminotransferase (ALT) and HBV DNA levels indicated HBV variation. When resistance mutations to LMV or ADV (rtM204V/I for LMV, rtA181T/V and rtN236T for ADV) appeared along with the virologic breakthrough, which normally happened in 6−12 months treatment, a conversion therapy was carried out. The HBV-RT segment was PCR-amplified using HBV DNA extracted from the serum sample as a template and sequenced. Sequence alignments with a reference indicated a variety of quasispecies in CHB patients after NA treatment, including rtS21A, rtL122F, rtN124F/H, rtP130Q, rtD131N, rtY135S/F, rtV142E, rtL145M, rtL180M rtA181V/T, rtS202G, rtM204V, rtF221Y, rtN236T, rtN238 R/H, rtT240Y, rtN248H, rtE263D and rtQ267H in the HBV-RT sequence (Figure 1) [31−33]. After LMV treatment, rtM204V and/or rtL180M mutations emerged as described in previous reports [12,16,17]. Interestingly, a novel mutation, rtQ267H/R, was occasionally found after LMV therapy. Other than rtA181V and rtN236T, which occurred in all samples after about 12 months of ADV administration, we also commonly found the mutations rtN238R/H, rtT240Y and rtN248H, even though they did not appear in all samples (Figure 1). The frequencies of rtN238 and rtN248 (80%) substitutions were higher than that of rtT240 (40%) in different patients (Figure 1). The relatively high prevalence of these three substitutions in CHB patients with ADV therapy implies that they might be selected after ADV long-term usage and attenuate the anti-HBV effect of ADV.

2.2 The replication capacity of MT pHBV1.3 *in vitro*

To further analyze the influence of the rtN238, rtT240 and rtN248 mutations on HBV replication and sensitivity to ADV, three MT HBV replication-competent plasmids, pHBV-rtN238R, -rtT240Y and -rtN248H, were constructed based on pHBV1.3 [34]. The replication-competent plasmids were then transfected into Huh7 cells and pHBV1.3 was used as a control. Southern blotting (upper panel) and subsequent densitometry analysis (lower panel) revealed no significant difference between the MT and parent HBV (Figure 2). There was a slightly lower HBV DNA level in MTs compared with the WT as determined by qRT-PCR, but they were not statistically different (Figure 2(b)). Thus, none of the substitutions had any obvious effect on HBV replication.

HBsAg and HBeAg levels in culture supernatant at 96 hpt were also measured by ELISA (Figure 2(c)), while HBcAg in cell lysates was analyzed by Western blotting (Figure 2(d)). The results indicated that the recombinant HBV with the substitution of rtN238R, rtT240Y or rtN248H, respectively, had similar expression levels as HBsAg, HBeAg and HBcAg with that of HBV WT. Taken together, the rtN238R, rtT240Y and rtN248H substitutions had no obvious effect on HBV DNA replication or gene expression under our experimental conditions.

2.3 rtN238R, rtT240Y and rtN248H in HBV substitutions are involved in reduced susceptibility to ADV

In order to determine whether rtN238R, rtT240Y and rtN248H influenced HBV susceptibility to LMV and ADV, Huh7 cells were transfected with MT or WT HBV plasmids, then exposed to the indicated NA concentrations and the core-associated DNA was analyzed by Southern blotting. As shown in Figure 3(a), LMV inhibited the replication of MT and WT HBV in a dose-dependent manner. In contrast, ADV reduced WT HBV replication in a dose-dependent manner, but did not influence the three MT HBVs, indicating that MTs were less susceptible to ADV (Figure 3(b)).

qRT-PCR targeted to HBV RC DNA was performed to calculate the increase in resistance of rtN238R, rtT240Y and rtN248H to ADV. The half maximal effective concentration

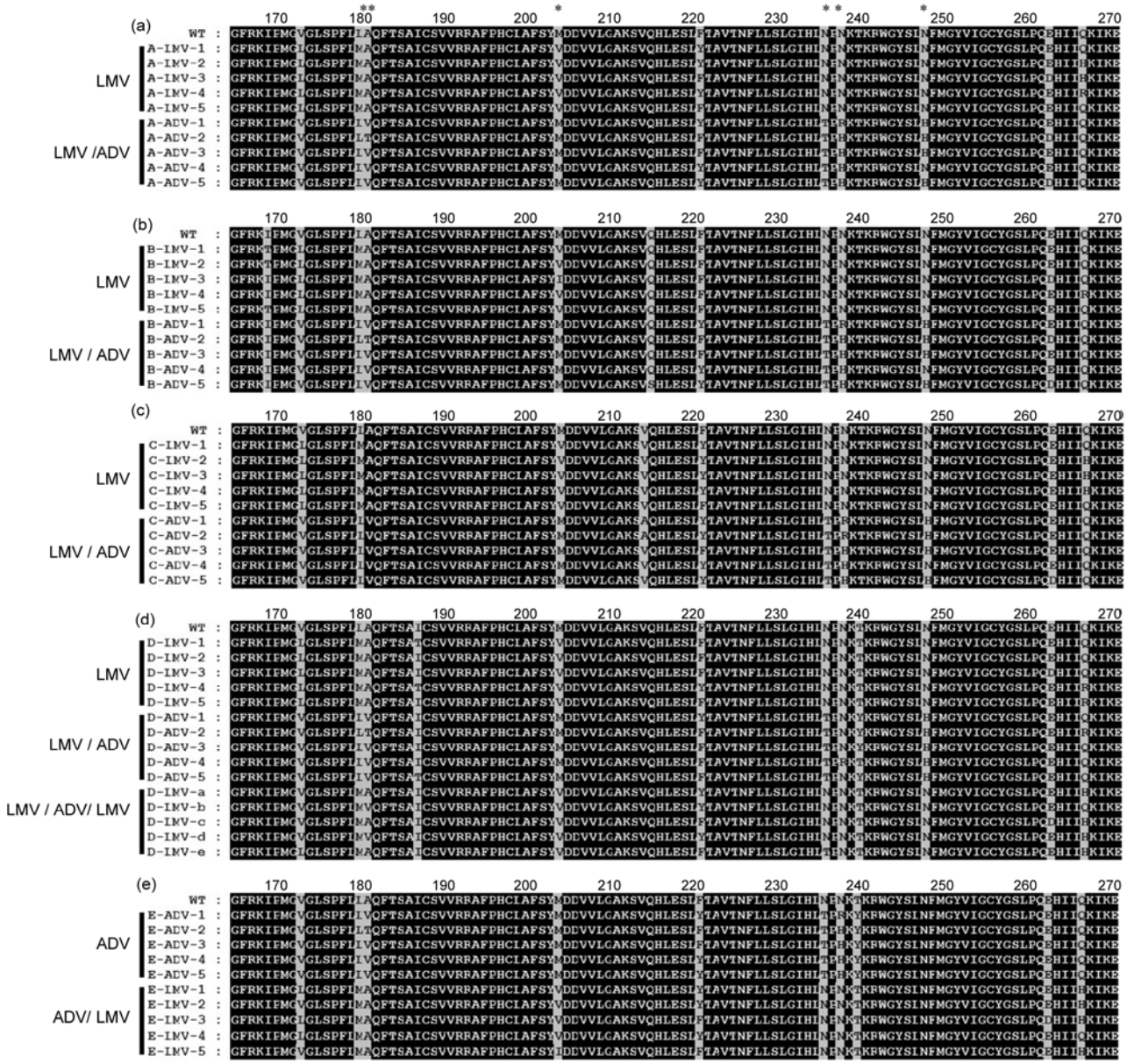

Figure 1 Sequence alignments of HBV RT regions. HBV DNA was isolated from serum samples of CHB patient (a), (b), (c), (d) and (e) who treated alternately with LMV and ADV when the drug-resistance showed up. The HBV-RT segments were PCR-amplified and cloned. Five clones were sequenced for each sample. Then the sequences were aligned with the reference sequence (WT, GenBank accession no. X02763.1, genotype A). The region (rt167 to rt270) contained the substitution sites are shown.

(EC_{50}) of pHBV1.3-WT, -rtN238R, -rtT240Y and -rtN248H to ADV were about 1.38, 7.49, 3.99 and 7.90 μmol L^{-1}, respectively, as calculated by qRT-PCR (Figure 4(b)), which was much higher than that of WT (1.38 μmol L^{-1}). Their resistance indexes (the EC_{50} value of MT divided by that of WT) to ADV were about 5.42, 2.89 and 5.72, respectively.

2.4 rtN238R, rtT240Y and rtN248H substitutions maintained normal susceptibility to other NAs, but not ADV

rtN238R, rtT240Y and rtN248H substitutions reduced the anti-HBV effect of ADV. We further analyzed their susceptibility to other NAs. Huh7 cells were transfected with pHBV-rtN238R, -rtT240Y, -rtN248H or pHBV1.3 and then treated with indicated concentrations of LMV, ETV, LdT and TDF. qRT-PCR was used to measure the HBV RC-DNA copy number and the EC_{50} of WT and MT HBV to LMV (Figure 4(a)), ADV (Figure 4(b)), LdT (Figure 4(c)), ETV (Figure 4(d)) and TDF (Figure 4(e)). The EC_{50} values of pHBV-rtN238R, -rtT240Y, -rtN248H to LMV, LdT, ETV and TDF were all close to that of WT (1.08, 11.5, 0.79 and 0.195 μmol L^{-1}, respectively). The results indicated that rtN238R, rtT240Y and rtN248H decreased the susceptibility

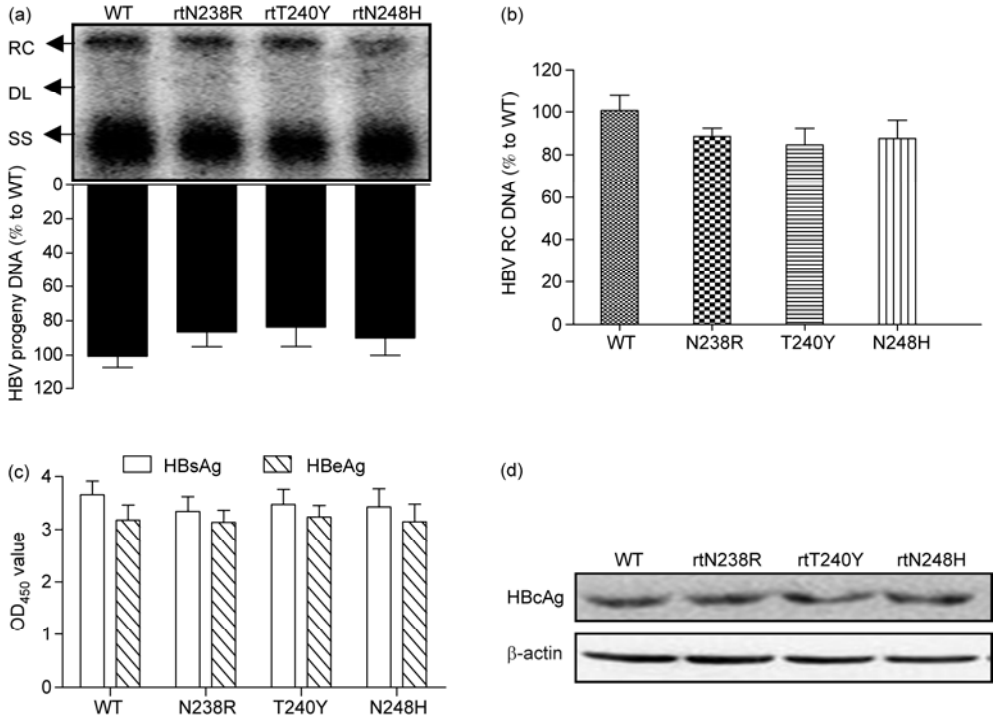

Figure 2 Replication capacity and antigen expression levels of WT and HBV mutants. (a) Huh7 cells were transfected with WT or MT HBV plasmids. Encapsulated viral DNA was extracted and detected by Southern blotting (upper panel). Relaxed circular (RC), double stranded linear (DL) and single stranded (SS) HBV DNAs are indicated. The relative level of WT HBV replication capacity is shown as a percentage of the control by gray analysis (lower panel). (b) HBV RC DNA of mutants compared with that of WT HBV is shown as a percentage of the control. Each value is the mean of at least 3 independent experiments. The error bars represent the standard deviation (SD). (c) HBsAg and HBeAg in the supernatant were detected using an ELISA diagnostic kit (Shanghai Kehua Diagnostic Medical Products Co., Ltd.) according to the manufacturer's instructions. (d) Total lysates of Huh7 cells were prepared and 50 μg of total protein was subjected to Western blotting. HBcAg was detected using mouse polyclonal antibody (upper panel) and β-actin was used as a loading control (lower panel).

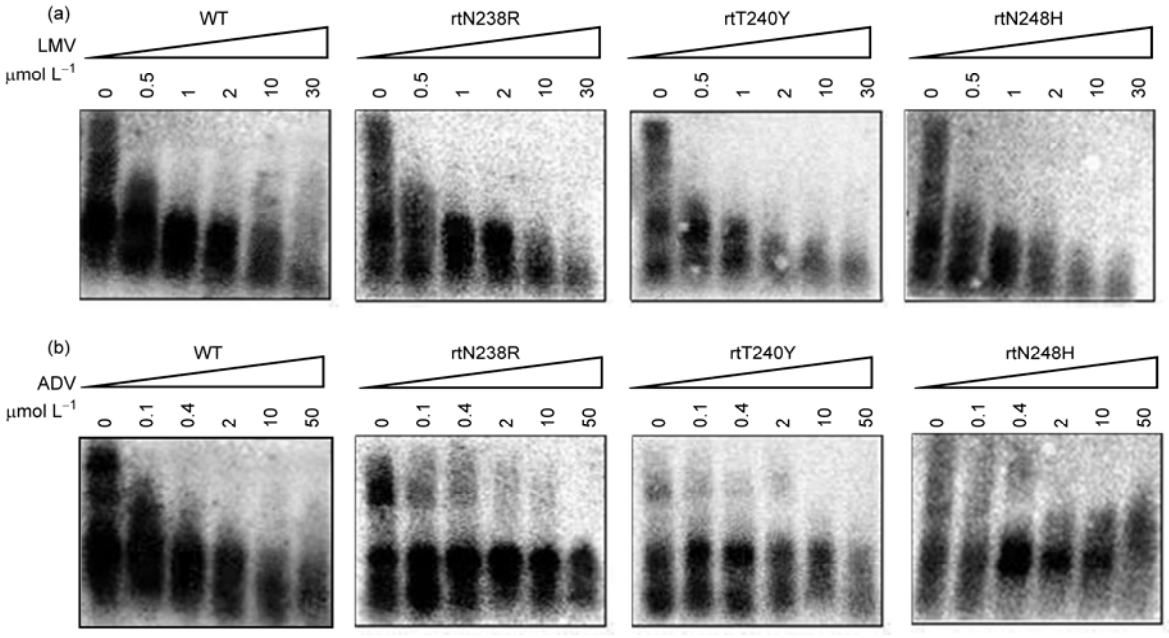

Figure 3 Southern blot analysis was used to detect the anti-HBV effect of LMV and ADV. Huh7 cells were transfected with pHBV1.3, pHBV-rtN238R, -rtT240Y and -rtN248H, and treated with LMV (a) and ADV (b) at the indicated concentrations. Core-associated HBV DNA was extracted from the cell lysates 96 hpt and subjected to agarose gel electrophoresis, followed by denaturation and Southern blotting.

of HBV to ADV, but they were still susceptible to LMV, LdT, ETV and TDF *in vitro*.

3 Discussion

HBV infection is a worldwide health problem. Chronic HBV infection can be treated with NAs, which are also widely used for HIV and herpes viruses [35,36], but drug resistance severely impaired HBV therapy. In the present study, we identified resistance mutations of HBV to ADV, which has been widely used together with LMV. Among the mutations found in patient blood samples, rtL180M, rtA-181V/T, rtS202G, rtM204V/I, and rtN236T were confirmed as NA resistance mutations, whereas rtS21A, rtL122F, rtN124F/H, rtP130Q, rtD131N, rtY135S/F, rtV142E, rtL145M, rtF221Y, rtE263D and rtQ267H were found in CHB patients [31−33,37,38] . Moreover, rtN238R, rtT240Y and rtN248H were regularly turned up in CHB patients with ADV treatment, and they were introduced into replication- competent plasmids to demonstrate that they were also responsible for reduced susceptibility to ADV *in vitro*.

NAs that mimic physiological nucleosides (dA/T/G/CTP) in terms of uptake and metamorphosis are incorporated into newly synthesized viral DNA giving rise to synthesis inhibition and termination [39]. ADV showed comparative antiviral activity against LMV-resistant HBV mutants and WT HBV [40]. However, in the first year of treatment, ADV resistance emerged in about 20% of LMV-resistant patients and in about 2% of patients with no LMV resistance-associated mutations [12,41], which may explain why so many ADV resistance-associated mutations were selected and found in the present study. A higher resistance equates to weaker selective pressure; hence, the frequency of rtN238R and rtN248H was higher than that of rtT240Y, in accordance with the clinical data. Overall, according to our data, HBs/e/cAgs were not directly associated with HBV replication *in vitro*.

HBV resistance mutations were all found in HBV-RT, with no exception for rtN238R (domain D), rtT240Y (domain D) and rtN248H (domain E). The HBV genome overlaps, as the *S* gene is completely contained within the *P* gene and mutations in the HBV polymerase coding region may confer NA resistance that usually affects the HBsAg. However, rtN238R, rtT240Y and rtN248H substitutions had no influence on HBsAg secretion, because they all reside after the stop codon of the *S* gene.

According to the three-dimensional (3D) HBV-RT structure predicted by SWISS-MODEL (http://swissmodel.expasy.org/), an automated protein modeling server, rtN238R, rtT240Y and rtN248H are situated close to residue M204 in the YMDD motif (data not shown), which was found to be the catalytic center for DNA synthesis. Substitutions may change the RT structure, and then prevent ADV from entering the catalytic pocket or make ADV separate from the catalytic pocket easyly thereby reduce the anti-HBV effect of ADV.

Fortunately, HBV mutants with any one of the rtN238R, rtT240Y or rtN248H substitutions retained normal susceptibility to LMV, LdT, ETV and TDF. In further research, it will be interesting to test whether the combination of these three mutations enhances ADV resistance, and what will

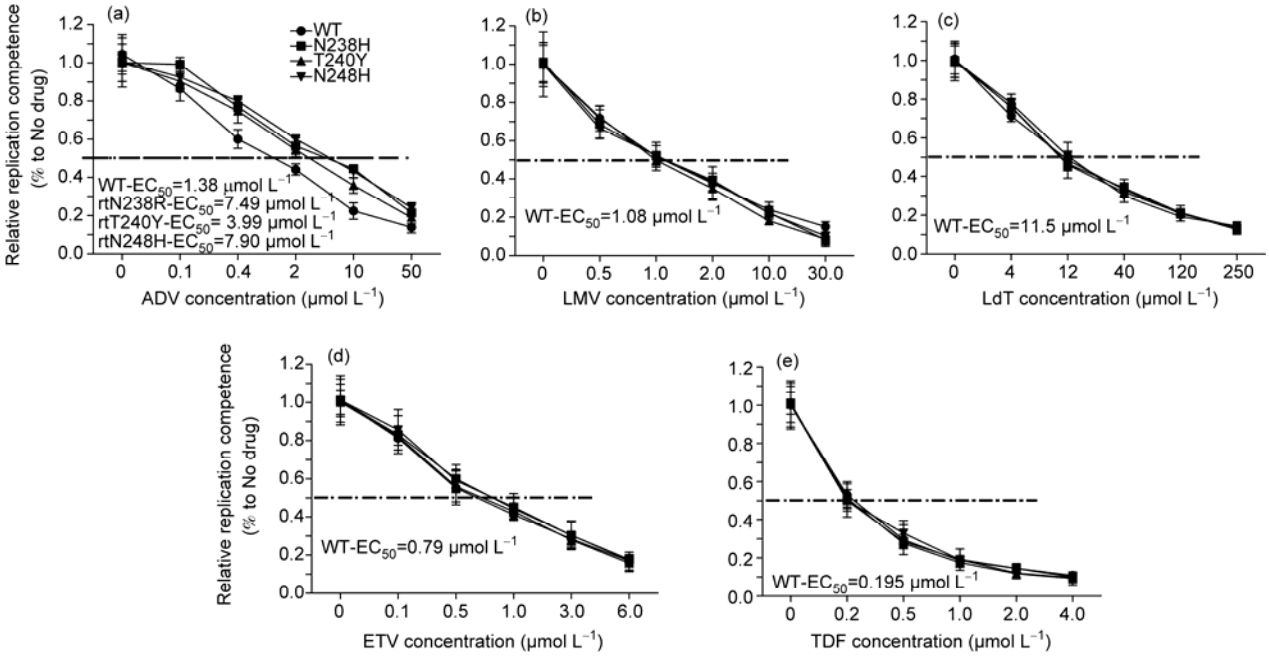

Figure 4 qRT-PCR was performed to detect the anti-HBV effect of 5 NAs. Huh7 cells were transfected with HBV-bearing plasmids, RC-DNA of encapsulated HBV DNA was extracted and analyzed by qRT-PCR to evaluate the antiviral effect of ADV (a), LMV (b), ETV (c), LdT (d) and TDF (e). The results shown were calculated as means ± 2 SDs (n=3).

happen when they are combined with rtA181V/T and/or rtN236T. Further, we plan to explore the susceptibility to NAs in hydrodynamically injected mice with replication-competent pAAV-HBV1.3.

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